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Article type : Regular Manuscript

FRS7 and FRS12 recruit NINJA to regulate expression of glucosinolate biosynthesis genes

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/nph.16586

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Received: *19 February 2020*

Accepted: *25 March 2020*

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Summary

- The sessile lifestyle of plants requires accurate physiology adjustments to be able to thrive in a changing environment. Plants integrate environmental timing signals to control developmental and stress responses.
- Here, we identified Far1 Related Sequence (FRS) 7 and FRS12, two transcriptional repressors that accumulate in short-day conditions, as regulators of Arabidopsis glucosinolate (GSL) biosynthesis.
- Loss of function of *FRS7* and *FRS12* results in plants with increased amplitudes of diurnal expression of GSL pathway genes. Protein interaction analyses revealed that FRS7 and FRS12 recruit the NOVEL INTERACTOR OF JAZ (NINJA) to assemble a transcriptional repressor complex.
- Genetic and molecular evidence demonstrated that FRS7, FRS12 and NINJA jointly regulate the expression of GSL biosynthetic genes, and thus constitute a molecular mechanism that modulates specialized metabolite accumulation.

Key words: Glucosinolate, jasmonate, diurnal, circadian, defense, immune responses, light, rhythms.

Introduction

Plants are sessile organisms that constantly need to adjust to changes in their environment. Chemical strategies of plants to defend against biotic stresses rely on constitutive and induced defense responses. The latter strategy consists of providing the appropriate chemical defense response at the correct time to minimize the use of energetic resources. Defense is mediated by a plethora of specialized metabolites that may act directly or indirectly to prevent threats (Howe & Jander, 2008). In *Brassicaceae*, glucosinolates (GSLs) constitute an important class of stress-induced metabolites accumulating in response to wounding or herbivores (Burow & Halkier, 2017; Sánchez-Pujante *et al.*, 2017). GSLs are nitrogen- and sulfur-containing compounds derived from amino acid precursors. Upon insect feeding or tissue damage, GSLs are hydrolyzed by myrosinases, generating an unstable aglycone that is able to rearrange into toxic thiocyanates, isothiocyanates or nitriles (Wittstock & Halkier, 2002). The GSLs biosynthesis pathways have been described in detail in *Arabidopsis thaliana* (Sønderby, I. E. *et al.*, 2010). These compounds are classified in three classes based on the origin of their amino acid side chain: aliphatics, benzenics and indoles (Fahey *et al.*, 2001; Sønderby, I. E. *et al.*, 2010). Each class specifically provides defense against particular herbivore species that might be present at a particular time of the day and/or the year (Hopkins *et al.*, 2009) As such, biosynthesis, transport and storage of each of these GSL classes need to be tightly coordinated to ensure that appropriate chemical defenses are present in the right tissue and at the right time.

Plant defense responses, including GSL biosynthesis, are regulated by complex hormonal networks controlling the balance between growth and defense (Pauwels *et al.*, 2009; Robert-Seilaniantz *et al.*, 2011; Huot *et al.*, 2014; Gimenez-Ibanez *et al.*, 2015; Havko *et al.*, 2016). Jasmonate and its derivatives (JAs) are such pivotal phytohormones controlling plant defense and developmental processes (Wasternack & Hause, 2013; Chini *et al.*, 2016; Goossens *et al.*, 2016; Wasternack & Strnad, 2019). For instance, a JA burst after wounding or herbivore attack triggers the activation of JA responses through a massive transcriptional reprograming of the cell, ultimately leading to the accumulation of defensive compounds, such as GSLs, on the one hand, and to growth repression on the other hand (Ehlting *et al.*, 2008; Pauwels *et al.*, 2009; Wasternack & Strnad, 2019). JA-elicited transcriptional reprogramming is partially dependent on the clade IVa basic helix-

loop-helix (bHLH) MYC transcription factors (TFs) MYC2, MYC3, MYC4 and MYC5 (Fernández-Calvo *et al.*, 2011; Niu *et al.*, 2011; Schweizer *et al.*, 2013; Song *et al.*, 2017). In an unelicited state, MYCs are repressed by a protein complex composed of the Jasmonate ZIM domain (JAZ) proteins, the Novel Interactor of JAZ (NINJA) and the Groucho/Tup1-type co-repressor TOPLESS (TPL) (Chico *et al.*, 2008; Pauwels *et al.*, 2010; Pauwels & Goossens, 2011; Chini *et al.*, 2016). Upon JA elicitation, signal transduction is regulated by a Skp1/Cullin/F-box (SCF) E3 ubiquitin ligase (COI1) that directly recognizes JA-isoleucine (Fonseca *et al.*, 2009; Zhang *et al.*, 2015; Nagels Durand *et al.*, 2016) and subsequently targets JAZ proteins for proteasomal degradation, thereby derepressing MYCs and thus activating defense responses (Chini *et al.*, 2007; Sheard *et al.*, 2010).

A growing body of evidence indicates that plant growth–defense trade-offs are synchronized according to environmental rhythms, such as daily cycles (Greenham & McClung, 2015; Song *et al.*, 2015). For example, Arabidopsis synchronizes JA-mediated defense to anticipate daily herbivore attack through a process depending on the circadian clock (Goodspeed *et al.*, 2012). In addition to the endogenous time keeper, environmental cues such as light quality also influence JA signaling. MYC TFs have been shown to be stabilized by light in a process mediated by the red- and blue-light photoreceptors phytochrome B (phyB), cryptochrome 1 (CRY1) and CRY2. Conversely, dark exposure promotes proteasomal degradation of MYC TFs through the action of the ubiquitin E3-ligase enzyme constitutive photomorphogenic1 (COP1) (Chico *et al.*, 2014). Furthermore, plants that grow in the shade upregulate the sulfotransferase 2a (ST2a) by the action of the phyB signaling pathway. This enzyme works to reduce the pool of active JA to a sulfated derivative of jasmonate, thereby prioritizing shade avoidance over defense under intense plant competition. (Fernández-Milmanda *et al.*, 2020). In relation, a recent work demonstrated the function of the Far1 Related Sequence (FRS) family proteins FAR-RED ELONGATED HYPOCOTYL3 (FHY3) and FAR-RED-IMPAIRED RESPONSE1 (FAR1) in balancing the growth–defense trade-off, by transcriptionally modulating JA signaling in shade conditions (Liu *et al.*, 2019). These FRS TFs physically interact with MYC2 under low red/far red light conditions to activate defense-related genes. Moreover, FHY3 and FAR1 recruit JAZ proteins to repress genes involved in plant growth. Here, we show that two additional FRS TFs, *i.e.* FRS7 and FRS12, physically

interact with the NINJA co-repressor to act as negative regulators of GSL biosynthetic genes. As such, our results suggest that the FRS7–FRS12–NINJA complex might regulate certain aspects of the Arabidopsis defense and specialized metabolism arsenal.

Materials and Methods

Plant material and growth conditions

All *A. thaliana* plants used in this study were in the Col-0 ecotype background. The *frs12-1* mutant was obtained from the SALK T-DNA collection (http://signal.salk.edu), corresponding to the SALK_030182 accession. The initial *frs7-1* mutant was in the Ws-2 background ecotype and was obtained from the INRA T-DNA insertion collection (http://www-ijpb.versailles.inra.fr/en/plateformes/cra/index.html), corresponding to the FLAG_196C09 accession. The single *frs7-1* and double *frs7-1*;*frs12-1* mutant in the Col-0 ecotype were obtained by crossing the parental lines, followed by backcrossing the progeny with the Col-0 background for three generations. The *ninja-1* (346c mutation) line was obtained from Edward Farmer (Acosta *et al.*, 2013), then crossed to the Col-0 wild type to cross out the *pJAZ10::GUSplus* reporter construct. The triple *frs7-1*;*frs12-1*;*ninja-1* line was obtained by the cross of *frs7-1*;*frs12-1* and the *ninja-1* parental lines. The *ProNINJA:NINJA-CITRINE (CIT)* line was previously described (Gasperini *et al.*, 2015). The *ProFRS7:FRS7-HA;ProNINJA:NINJA-CIT* and *ProFRS12:FRS12-HA;ProNINJA:NINJA-CIT* lines were generated by crossing the *ProFRS7:FRS7-HA and ProFRS12:FRS12-HA* lines (Ritter *et al.*, 2017) with the *ProNINJA:NINJA-CIT* line.

Yeast two-hybrid experiments

The entry vectors were used for LR reactions (Invitrogen) with pGADT7 and pGBKT7 (or pGBT9) yeast two-hybrid vectors, generating bait and prey constructs. NINJA domain constructs were previously generated (Pauwels *et al.*, 2010). The N-terminal region of *FRS12* was generated by PCR amplification and subsequent cloning of the first 921 base pairs with the addition of a stop codon at the end the coding sequence. The C-terminal region of *FRS12* was generated by PCR amplification and subsequent cloning of the 922-2365 bp region with the addition of a start codon at the beginning of the sequence (See Table S1 for the primers used). Bait and prey plasmids were co-transformed in the *Saccharomyces cerevisiae* strain PJ69-4A (Cuéllar Pérez *et al.*, 2013). Transformed

yeast colonies were selected on dropout medium -Leu and -Trp. Protein interactions were assessed by growing yeast colonies on selective medium -Leu, -Trp and -His for 3-4 days at 30°C. Three independent colonies were grown for each combination. Yeast strains co-transformed with the empty pGADT7 and pGBKT7 vectors were used as negative control.

Bi-molecular fluorescence complementation (BiFC)

For BiFC, the *FRS7, FRS12* and *NINJA* were Gateway-recombined to generate amino-terminal fusions to the nGFP or cGFP tags using the pH7m24GW2 or pKm24GW2 vectors, respectively. The nGFP and cGFP constructs were transiently expressed by *A. tumefaciens*-mediated transformation of lower epidermal leaf cells of 3- to 4-week-old *N. benthamiana* plants using an infiltration buffer composed of 10 mM $MgCl_2$, 10 mM MES and 100 µM acetosyringone, and addition of a P19-expressing Agrobacterium strain to boost protein expression. All Agrobacterium strains were grown for two days, diluted to O.D. 600nm = 1 in infiltration buffer and incubated for 2 h at room temperature before mixing in a 1:1 ratio with other strains for injection. Three days after injection, interaction of the proteins was scored by screening lower epidermal cells for fluorescence using a Zeiss LSM 710 confocal microscope at X25 magnification.

Tandem affinity purification (TAP)

For TAP and tandem chromatin affinity purification, NINJA was Gateway-recombined in the pKCTAP vector to create a C-terminal fusion to the GS tag. TAP experiments were performed on Arabidopsis cell cultures (PSB-D) with protein G- and streptavidin-binding peptide (GS)-tagged bait. Protein interactors were identified by mass spectrometry using an LTQ Orbitrap VELOS and non-specific background proteins were filtered out as previously described (Van Leene *et al.*, 2015). Two biological replicates were analyzed.

Co-immunoprecipitation assay

Co-Immunoprecipitation was carried out in transgenic lines expressing either *ProFRS7:FRS7-HA* and *ProNINJA:NINJA-CIT* or *ProFRS12:FRS12-HA* and *ProNINJA:NINJA-CIT.* Sterilized seeds were placed on a nylon mesh filter (100-µm pore size) on MS-agar plates, and stratified in the dark for 2 to 3 days at 4°C. After

stratification, lines were grown in SD conditions for 15 days. A day before the experiment, lines were transferred to 20 mL liquid MS solution. The following day at zeitgeber time (hours after illumination) 16 (ZT16), seedlings were treated with 100 µM of cycloheximide for 1 h, then mock-treated or elicited with 50 µM of JA for 15 or 30 min. For protein extraction, a total of 2 g of seedlings were snap-frozen and ground in liquid nitrogen and suspended in 0.7 mL/g of HB^+ buffer. Samples were centrifuged and supernatants were quantified using the Bradford assay (Bio-Rad). A total of 2 mg of protein extract per sample was added to 20 µL GFP-trap beads (ChromoTek), then followed by 5 washes with 600 µL of HB^+ buffer. Samples were denatured in Laemmli buffer and run on a 4–15% TGX gel (Bio-Rad) for 20 min at 300 V, and blotted on a 0.2-µm PVDF membrane (Bio-Rad).

Immunoblotting

Total protein was extracted using HB^+ extraction buffer (25 mM Tris/HCl pH 7.6, 15 mM $MgCl_2$, 150 mM NaCl, 15 mM p-nitrophenyl phosphate, 60 mM β-glycerophosphate, 0.1% NP-40, 0.1 mM Na3VO4, 1 mM NaF, 1 mM PMSF, 1 µM E64, complete proteinase inhibitor (Roche), 5% ethylene glycol) and protein concentration determined using the Bradford assay (Bio-Rad). Samples were denatured in Laemmli buffer and run on a 4–15% TGX gel (Bio-Rad) for 20 min at 300 V, and blotted on a 0.2-µm PVDF membrane (Bio-Rad). Antibodies used were anti-HA (3F10, Roche), anti-GFP (ab290, Abcam), anti-PSTAIR (SC-53, Santa Cruz) and anti-actin 8 (A0480, Sigma). Chemiluminescent detection was performed with Western Bright ECL (Isogen, http://www.isogen-lifescience.com/).

GUS histochemical analysis

Histochemical GUS staining was performed in 14-day-old homozygous seedlings expressing *ProFRS7:GUS-GFP, ProFRS12:GUS-GFP* or *ProNINJA:GUSplus* germinated under short-day (SD) conditions as described (Ritter *et al.*, 2017).

RNA isolation, cDNA preparation, qPCR and RNA-Seq analysis

RNA was extracted using the RNeasy Plant Mini Kit (Qiagen), and treated with DNase I (Promega) prior to complementary DNA (cDNA) synthesis with the iScript cDNA

Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Relative transcript abundance of selected genes was determined using the Roche LightCycler 480 system and the LC480 SYBR Green I Master Kit (Roche Diagnostics). Three technical repeats were considered (see Table S1 for the primers used). The amplification data were analyzed using the second derivative maximum method, and resulting cycle threshold values were converted into relative expression values using the comparative cycle threshold method.

For RNA-Seq analyses, wild-type, *frs7-1;frs12-1*, *ninja-1* and *frs7-1;frs12-1;ninja-1* lines were sown on MS-agar plates and stratified for 3 days at 4°C. After stratification, seedlings were grown in SD conditions for 10 days and harvested at ZT8 or ZT16. Three biological replicates were generated. RNA samples were processed by first preparing a Truseq (Illumina) stranded mRNA library, sequenced using NextSeq 500 70-bp single read and then sequenced at 30 million reads using Illumina NexSeq500 technology. Read quality trimming and filtering were performed with the FASTX-toolkit (v 0.0.13) with default settings. CutAdapt (v1.9.1) was used for adapter removal. Cleaned reads were aligned to the *A. thaliana* (TAIR10 version) genome using GSNAP (v 2013-06-27), allowing a maximum of five mismatches and default settings otherwise. Afterwards, the mapped reads were summarized on the gene level using htseq-count from the HTSeq python package. All above analyses were carried out using the Galaxy (Afgan *et al.*, 2018) instance hosted at the VIB-UGent Center for Plant Systems Biology. Data were normalized using TMM, and common dispersion was estimated using the conditional maximum likelihood method implemented in edgeR (Robinson *et al.*, 2010). Differentially expressed genes were defined by a twofold difference between samples with corrected $P<0.05$ at a $FDR<0.05$. GO enrichment analysis was carried out with the PANTHER software (http://pantherdb.org/about.jsp). Genes presenting an $LFC>1.5$ ($P<0.05$ at a $FDR<0.05$) in at least one line were considered for hierarchical clustering analysis (HCA). HCA was performed on normalized data using MeV 4.9 (Eisen *et al.*, 1998) through Pearson Unicentered correlation.

Glucosinolate profiling

GSL quantification was performed by ultrahigh performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOFMS) according to a previously described protocol (Glauser *et al.*, 2012).

Y1H screen

The Y1H method used for GLS promoter screening has been described previously (Li *et al.*, 2014). Briefly, PCR primers were designed to amplify about 2000 bp of each of the aliphatic GLS promoter upstream of the predicted translational start codon or the promoter sequence to the start of the next gene(Li *et al.*, 2014). The promoters (*GSOX5, CYP79F1, SOT5C, UGT74B, IMD3, GSOX2, GSOX3, GSOX4, GSOX1, BZO1, GSTU20, CSLyase, GSOH, GSTF9, UGT, IPMI2, GSTF11, BCAT4, BCAT3, IPMI1, AOP3, AOP2, BAT5, CYP83A1, CYP83B1, OMT, PMSR2, PMSR3, MYB29, MYB76, MAM1, MAM3, MYB28* and *PMSR1*) were amplified by Phusion High-Fidelity DNA Polymerase (Biolabs, M0530S) and cloned into pENTR 5' TOPO TA Cloning vector (Invitrogen, K591-20) (Li *et al.*, 2014). The promoters were then transferred into the pMW2 and pMW3 destination vectors by LR Clonase II Reaction (Invitrogen, 11791-019) and confirmed by DNA sequencing (Gaudinier *et al.*, 2011). The open reading frame of FRS7 and FRS12 in pENTR were obtained from Pruneda Paz et al. 2014 and recombined with pDESTAD-2u using LR Clonase II (Invitrogen) before transformation into yeast strain Yα1867 (Gaudinier *et al.*, 2011). All of the GLS bait promoters were screened against the prey TFs using the Y1H protocol as previously described (Gaudinier *et al.*, 2011; Taylor-Teeples *et al.*, 2011; Li *et al.*, 2014).

Data availability

The RNA-Seq data sets are available at the NCBI Sequence Read Archive sequence database under accession codes E-MTAB-7397 for the experiment comparing wild type to *frs7-1;frs12-1* at ZT8 and ZT16; and E-MTAB-7402 for the experiment comparing wild type to *frs7-1;frs12-1*, *ninja-1* and *frs7-1frs12-1;ninja-1* at ZT8. The authors declare that all other data supporting the findings of this study are available within the manuscript and its supporting information files or are available from the corresponding author upon request.

Results

FRS7 and FRS12 regulate defense response genes

We have previously reported on FRS7 and FRS12 as transcriptional repressors of photoperiodic growth and flowering time, and suggested their potential implications in other processes, including stress-related mechanisms (Ritter *et al.*, 2017). To further explore the functions of these proteins, we carried out a transcriptome analysis by RNA-Seq of SD-grown wild-type and *frs7-1*;*frs12-1* double knocked-out (KO) plants harvested at two times of the day, *i.e.* at ZT8 before lights went off and ZT16 in darkness. In agreement with the previously described function of FRS7 and FRS12 in photoperiod regulation, functional classification by gene ontology (GO) of the differentially expressed genes (DEGs) highlighted the deregulation of seed and reproductive structure development, photosynthesis and defense (Fig. 1a; Fig. S1). A significant portion (77%) of DEGs in the *frs7-1*;*frs12-1* line showed a time-specific deregulation at both ZT8 and ZT16, suggesting a function for FRS7 and FRS12 in diurnal gene expression (Tables S2, S3). Because FRS7 and FRS12 are transcriptional repressors (Ritter *et al.*, 2017), we focused on the upregulated genes in the *frs7-1*;*frs12-1* line. In agreement with our previous findings (Ritter *et al.*, 2017), this list included several genes related to growth and/or diurnal rhythms. However, we noticed that also a large set of defense-related genes was upregulated, and notably those involved in GSL biosynthesis (Fig. 1a).

Because of the strong induction of the GSL pathway in the *frs7-1*;*frs12-1* line, we next tested possible redundancies between FRS7 and FRS12 by RT-qPCR analysis of the single *frs7-1* and *frs12-1* lines and the double *frs7-1;frs12-1* line grown in SD conditions at ZT8. Although a clear upregulation trend was observed for the genes *BCAT4*, *MAM1, CYP79F1* and *CYP83A1* in the double *frs7-1;frs12-1* line compared to the wild type, this experiment showed statistically significant differences only for the *BCAT4* and *CYP83A1* genes (Fig. S2a). In contrast, single *frs7-1* or *frs12-1* mutant lines did not show any differences compared to the wild type in terms of steady-state transcript levels of any of these genes (Fig. S2a). Given that GSL pathway genes rhythmically oscillate over the daily cycle (Covington *et al.*, 2008), phasing at evening time, we questioned whether the *frs7-1;frs12-1* mutant would present altered expression patterns of these genes during the diurnal cycle. Time-course studies in SD conditions showed rhythmic expression peaks with increased amplitudes for *CYP79F1*, *CYP83A1* and

MAM1 in the *frs7-1;frs12-1* line compared to wild-type plants (Fig. 1b). These results suggest that FRS7 and FRS12 function as evening repressors of GSL pathway genes. To further support the coordinated function of FRS7 and FRS12 in the diurnal regulation of the GSL pathway, we assessed a second *frs7;frs12* (*CRISPR #3-11*) double mutant that was previously generated using the CRISPR-CAS9 technology (Ritter *et al.*, 2017). This line was grown under SD conditions together with the *frs7-1;frs12-1* line and the wild type, and harvested at ZT4 and ZT8. Although no significant differences in the expression of *CYP79F1* and *CYP831* were observed at ZT4, both double mutant lines showed significant inductions of these genes at ZT8 compared to the wild type (Fig. S2b). Together, these results support a redundant action of FRS7 and FRS12 in the repression of GSL biosynthetic genes in a time of day-dependent manner.

The FRS7 and FRS12 proteins recruit the co-repressor NINJA

Recent work has demonstrated that the FRS family proteins FHY3 and FAR1 are able to modulate expression of Arabidopsis defense genes by physically interacting with transcriptional regulators involved in JA signaling, such as the JAZ and MYC2 proteins (Liu *et al.*, 2019). We accordingly evaluated by yeast two-hybrid (Y2H) analysis if FRS7 and/or FRS12 could equally regulate the expression of defense genes through physical interaction with proteins of the core JA signaling pathway and/or known transcriptional regulators of GSL biosynthesis. This analysis revealed that FRS7 and FRS12 can interact with JAZ4, JAZ6 and JAZ8, but also with NINJA (Fig. 2a). None of the tested MYC or MYB TFs interacted with either FRS7 or FRS12, at least not in our Y2H assays. The interaction of JAZ proteins and NINJA with FRS7 and FRS12 implicates these TFs in JA signaling, and consequently further in the regulation of plant defense.

NINJA was shown to be essential in defense signaling, not only because of its capacity to co-repress the MYC TFs, but also through its interaction with ERF19 (Huang *et al.*, 2019). These results, together with our Y2H analysis, indicate that NINJA is capable of recruiting a diverse set of additional proteins to regulate plant defense outside the core JA signaling module. To further explore the NINJA interactome, we used an advanced tandem affinity purification-mass spectrometry (TAP-MS) method with improved MS sensitivity compared to our previous study (Pauwels *et al.*, 2010; Van Leene *et al.*, 2015). NINJA was fused C-terminally to a TAP-tag and expressed under the

CaMV35S promoter (*Pro35S*) in *A. thaliana* cell cultures. This improved TAP method expanded our previous NINJA interactome identifications with additional components of the core JA signaling module (Fig. 2b; Table S4). These included the JAZ proteins JAZ2 and JAZ12, the co-repressor TPL, which are all direct interactors of NINJA (Pauwels *et al.*, 2010), as well as the bHLH factors MYC2, MYC3 and MYC4, which interact directly with the JAZ proteins (Fernández-Calvo *et al.*, 2011). Furthermore, we found other ZIM-domain proteins such as PEAPOD2 (PPD2) that also contains the TIFY motif responsible for interaction with NINJA (Pauwels *et al.*, 2010). The involvement of the PPD2–NINJA complex in the coordination of growth and the regulation of lamina size and curvature has recently been shown, illustrating the additional function of NINJA in leaf development (Baekelandt *et al.*, 2018). Finally, this advanced TAP analysis also identified FRS12 as interactor of NINJA, corroborating our Y2H results. Despite the latter, TAP with NINJA however did not retrieve any of the previously identified interactors of FRS12 and FRS7, such as AT-HOOK MOTIF NUCLEAR-LOCALIZED PROTEIN 14 or HISTONE-LIKE 4 (Ritter *et al.*, 2017). This result suggests that NINJA, FRS7 and FRS12 could operate in a protein complex that is not related to that comprising the previously identified interactors of FRS7 or FRS12.

Because the previous TAP method repeatedly applied on distinct members of the JAZ protein family did not reveal FRS7 or FRS12 as interactors (Fernández-Calvo *et al.*, 2011; Pauwels *et al.*, 2015), we decided to focus our further investigation first on the possible significance of the NINJA–FRS7 and NINJA–FRS12 interactions in plant defense signaling. We further investigated the physical interactions in more detail. Bimolecular fluorescence complementation (BiFC) assays in *Nicotiana benthamiana* leaves showed only a nuclear-localized GFP signal when nGFP-NINJA was transiently co-expressed either with cGFP-FRS12 or cGFP-FRS7 (Fig. 2c; Fig. S3) and thereby demonstrated the co-localization of FRS7, FRS12 and NINJA in the nucleus and corroborated the physical interactions of FRS7–NINJA and FRS12–NINJA. Next, to evaluate if other members of the FRS family could additionally interact with NINJA, we tested by Y2H the interactions with six other members of the FRS family, including FHY3 and FAR1. This analysis, added to the TAP results, indicated that NINJA preferentially recruits FRS7 and FRS12 among the members from the FRS family (Fig. 2b-d). NINJA, in turn, belongs to the family of ABSCISIC ACID INSENSITIVE 5 (ABI5)-binding proteins

(AFPs) encompassing four other members and sharing three conserved domains, namely EAR, B and C (Garcia *et al.*, 2008; Pauwels *et al.*, 2010). A Y2H screen was carried out to evaluate if FRS7 or FRS12 could bind to other AFPs. Neither AFP2 nor AFP3 could interact with FRS7 or FRS12, suggesting that NINJA is the only member of the AFPs capable of interacting with FRS7 and FRS12 (Fig. S4).

With its C- and EAR-domains, NINJA acts as a molecular bridge connecting the JAZ proteins to the co-repressor TPL (Pauwels *et al.*, 2010). A Y2H assay of NINJA deletion series revealed the C-domain as necessary and sufficient to recruit FRS12, designating this domain as responsible for the interaction, which is also the case for the interaction with the JAZ proteins (Fig. 2e). Conversely, a deletion series of FRS12 revealed its C-terminal region to be responsible and sufficient for the interaction with the NINJA C-domain (Fig. 2f). This region contains a SWIM domain, that was previously designated as essential for the transcriptional regulatory and protein interaction activities of FHY3 (Lin *et al.*, 2008). Taken together, these results define FRS7 and FRS12 as non-JAZ proteins capable of interacting with the C-domain of NINJA.

NINJA is co-expressed with *FRS7* and *FRS12*

Next, we investigated if *FRS7*, *FRS12* and *NINJA* share spatial and temporal expression patterns, and thus can potentially build repressor complexes *in planta*. We examined the spatial expression of *NINJA* in seedlings expressing the *β-glucuronidase plus* (*GUSplus*) reporter gene driven by the *NINJA* promoter (*ProNINJA:GUS*) and compared them to seedlings expressing *ProFRS7:GUS* and *ProFRS12:GUS* reporter lines, all grown in SD conditions (Fig. S5). GUS staining revealed that *NINJA* is transcribed along the root meristem (RM) and vasculature, and the leaf primordia, vasculature, lamina and tips. The *ProFRS7:GUS* line reflected *FRS7* transcription along the RM, the shoot apical meristem (SAM), and the leaf primordia, vasculature and lamina. The *ProFRS12:GUS* line indicated *FRS12* transcription along the RM, the SAM, the leaf primordia, and throughout the seedling vasculature. Together, these results illustrate the co-localized expression of *NINJA*, *FRS7* and *FRS12* at the RM, the leaf primordia and the leaf vasculature. Next, we inquired whether the time of the day could affect NINJA, FRS7 or FRS12 accumulation at the protein level. With this aim, we followed FRS7, FRS12 and NINJA protein abundances in SD-grown seedlings expressing these proteins fused to epitope tags

under the regulation of their native promoters. Protein levels of the three proteins remained relatively stable throughout the diurnal cycle (Fig. 3a, Fig. S6).

Having established that *NINJA*, *FRS7* and *FRS12* are temporarily and spatially co-expressed, thus co-occurring *in planta*, we finally verified if these proteins eventually assemble into a complex *in planta*. Therefore, we generated lines expressing *NINJA-CIT* and *FRS7-HA* or *FRS12-HA* under the regulation of their native promoters. We tested by co-immunoprecipitation if interactions of these proteins in a complex occurred in SD conditions, and also if JA impinges on the stability of the complex. These lines were first grown in SD conditions and then treated at ZT7 with mock or JA for 15 or 30 min. In mock-treated plants, FRS7-HA and FRS12-HA co-precipitated with NINJA-CIT (Fig. 3b). In contrast, the αPSTAIR control protein did not co-precipitate with NINJA-CIT, demonstrating the specific interaction of NINJA *FRS7*–FRS7 and NINJA–FRS12 under the tested conditions. JA treatment for 15 or 30 min resulted in a slight increase in the intensity of the FRS7-HA band, suggesting that NINJA–FRS7 interaction could be augmented under these conditions. However, JA treatment did not affect the intensity of co-immunoprecipitated FRS12-HA. Taken together, these results indicate that NINJA, FRS7 and FRS12 are spatially and temporally co-expressed and build protein complexes *in planta* in SD conditions.

FRS7, FRS12 and NINJA jointly regulate the expression of GSL pathway genes

To further understand the function shared by FRS7, FRS12 and NINJA, we generated a triple KO line by crossing the *ninja-1* and *frs7-1;frs12-1* lines and performed transcriptome analysis on it, as well as on the *frs7-1;frs12-1*, *ninja-1* and wild-type lines in comparable conditions to our initial transcriptome analysis (10-day-old seedlings grown in SD conditions and harvested at ZT8). The obtained transcriptome profiles were compared by HCA. Differentially expressed genes presenting a LFC>1 and a FDR<0.05 in at least one of the three lines were considered for further analysis, comprising 1,211 genes. Fourteen main clusters with distinctive expression profiles could be distinguished (Fig. S7; Table S5). Clusters comprising repressed genes (i.e. clusters 9-14) displayed an overrepresentation of genes involved in stress-related responses. For instance, cluster 10 grouped genes with a strong repression in all three lines and an overrepresentation of GO terms related to abscisic acid catabolism. Cluster 13 grouped

genes showing repression in all three lines with an enhanced repression in the *frs7-1;frs12-1;ninja-1* line, suggesting the additive deregulation of these genes by FRS7, FRS12 and NINJA. Functional GO classification of this cluster highlighted the functions of these genes in response to freezing, oil body biogenesis, defense and stress responses. Cluster 14 gathered genes downregulated in the *frs7-1;frs12-1* and *frs7-1;frs12-1;ninja-1*, but not in *ninja-1*, suggesting their dependence on FRS7 and FRS12. Genes in this cluster showed significant over-representation in immune responses dependent on salicylic acid and/or ethylene pathways. Cluster 6 gathered genes mainly upregulated in *ninja-1* and *frs7-1;frs12-1;ninja-1*, suggesting their dependence on NINJA. Genes in this cluster classified in JA biosynthesis and triterpenoid metabolism and corroborated previous transcriptome analysis of the *ninja-1* mutant (Acosta *et al.*, 2013; Gasperini *et al.*, 2015).

Because FRS7, FRS12 and NINJA act as transcriptional repressors, we further focused on those clusters showing common upregulation in *frs7-1;frs12-1*, *ninja-1* and *frs7-1;frs12-1;ninja-1.* Cluster 5 grouped genes upregulated in these three lines, with an enhanced induction in *frs7-1;frs12-1;ninja-1* (Fig. 4a, Fig S7). Functional classification of this cluster highlighted genes regulated by JA, in particular those belonging to GSL biosynthesis. The strong induction of these genes in the *frs7-1;frs12-1;ninja-1* triple mutant further supported the existence of an additive regulatory network for GSL biosynthetic genes involving FRS7, FRS12 and NINJA, but also additional transcriptional regulators, for instance the MYCs that indirectly interact with NINJA through JAZ. We further explored this postulation through time-course studies, given that both JA and GSL metabolism are reported to be under circadian regulation (Goodspeed *et al.*, 2012; Huseby *et al.*, 2013). We particularly questioned whether *ninja-1*, *frs7-1;frs12-1* and *frs7-1;frs12-1;ninja-1* mutants would present altered expression patterns of these genes in SD conditions. Time-course analysis indeed revealed rhythmic expression of *CYP83A1* and *CYP79F1* in all lines, with a phase shift of 4 h later in the *frs7-1;frs12-1, ninja-1* and *frs7-1;frs12-1;ninja-1* lines compared to wild-type plants (Fig. 4b). Furthermore, the triple *frs7-1;frs12-1;ninja-1* mutant showed similar expression profiles as the *frs7-1;frs12*-1 and *ninja-1* mutants, but with increased amplitudes as compared to its parental lines. These findings further support the additive diurnal regulation of GSL biosynthetic genes by FRS7, FRS12 and NINJA.

FRS7 and FRS12 physically bind to promoters of GSL pathway genes in yeast

The observed deregulation in diurnal expression of GSL biosynthetic genes in the *frs7-1;frs12-1;ninja-1* mutant prompted us to analyze if FRS7 or FRS12 could physically bind to the promoters of these genes. To this end, we first mined the available FRS12 TChAPseq dataset for genes physically bound by FRS12 (Ritter *et al.*, 2017). However, neither GSL biosynthetic genes nor GSL transcriptional regulators were detected in this dataset. These observations could suggest the indirect regulation of the GSL pathway by FRS12, the binding to these promoters through FRS7 or the binding of FRS7 and FRS12 in different conditions to those previously tested. We therefore decided to further explore the possible binding of FRS7 and/or FRS12 to 34 promoters of the GSL pathway (see Materials and Methods) in a binary yeast one-hybrid (Y1H) screen. This analysis revealed the binding of FRS7 and FRS12 to 21 GSL pathway gene promoters, including those involved in both indolic and aliphatic GSL pathways (Fig. 4c). Interestingly, the FRS7 bound to 19 promoters, whereas FRS12 bound to only 4 promoters, suggesting the accentuated importance of FRS7 for binding promoters of GSL pathway genes. We then combined this analysis to available transcriptome data of an *FRS12* overexpression line(Ritter *et al.*, 2017) and the *frs7-1;frs12-1* mutant, to point out genes potentially bound and transcriptionally regulated by FRS7 and/or FRS12. These included for FRS7 the *IPM1*, *IPMI2*, *BAT5*, *PGL5* and *GSTF11* genes and for FRS12, the *MAM1* gene. Together, these findings further suggest the function of FRS7 and FRS12 as transcriptional regulators of GSL biosynthetic genes, however additional *in planta* experiments are required to confirm these observations.

FRS7–FRS12–NINJA complex(es) regulate GSL accumulation in short days

Given the regulation of GSL biosynthesis by FRS7, FRS12 and NINJA at the transcript level, we also assessed whether the GSL metabolite composition was affected in the *frs7-1;frs12-1, ninja-1* and *frs7-1;frs12-1;ninja-1* mutants. We analyzed a total of 21 compounds belonging to the indolic and aliphatic GSL classes in SD-grown plants harvested at ZT8 (Table S6). Statistical analysis of the metabolite profiles designated 16 GSL compounds as significantly modified among the mutant lines, suggesting that *FRS7–FRS12–NINJA* deregulation alters GSL composition (Fig. 5a). Particularly, a

significant increase of indolic GSL metabolites was observed in *ninja-1, frs7-1;frs12-1* and *frs7-1;frs12-1;ninja-1* compared to wild-type plants (Fig. 5b). Furthermore, the indolic GSL compounds glucobrassicin, 4-hydroxyglucobrassicin and 4-methoxy-glucobrassicin were increased with similar folds in both the *ninja-1* and *frs7-1;frs12-1* lines as compared to wild-type plants, and even further in an additive manner in the *frs7-1;frs12-1;ninja-1* line. Taken together, these results suggest that NINJA interacts with FRS7 and FRS12 to form protein complex(es) that regulate GSL biosynthesis.

Discussion

Plants synchronize their physiology with the diurnal cycle, which ensures an optimal allocation of resources at the correct time of the day. Daily changes are anticipated by the circadian clock, which integrates external light cues to control the periodic occurrence of its output processes. (Battey, 2000; Bendix *et al.*, 2015; Greenham & McClung, 2015; Shim *et al.*, 2017). Many specialized metabolites constitute clock outputs and thus oscillate during the day, *e.g.* to anticipate predictable herbivore feeding activity, which optimizes resource allocation between growth and defense (Greenham & McClung, 2015). The GSLs are pivotal metabolites for the defense system of plants from the *Brassicaceae* and related families of the order *Capparales* (Brown *et al.*, 2003; Meldau *et al.*, 2012; Baldwin & Meldau, 2013; Burow & Halkier, 2017; Sánchez-Pujante *et al.*, 2017). It was already known that many of the genes encoding GSL biosynthetic enzymes are directly regulated by the JA-modulated MYC TFs (Schweizer *et al.*, 2013). The MYC TFs act cooperatively with MYB TFs to bind the promoters and activate transcription of both indolic and aliphatic GSL pathway genes (Sønderby *et al.*, 2007; Sønderby, Ida Elken *et al.*, 2010; Frerigmann & Gigolashvili, 2014; Aarabi *et al.*, 2016; Seo & Kim, 2017). In relation to GSL regulation, JA contents oscillate during the day to peak at midday through a process that depends on the circadian clock (Goodspeed *et al.*, 2012). Consequently, the transcription of JA-responsive genes, such as those involved in GSL biosynthesis, also follows diurnal expression patterns (Covington *et al.*, 2008). In turn, GSLs can act as zeitgeber cues to reset the clock's pace (Kerwin *et al.*, 2011).

Previously, we demonstrated the function of FRS7 and FRS12 as transcriptional repressors of circadian clock outputs such as growth or flowering time (Ritter *et al.*, 2017). Here, we identified an additional function of these TFs in the modulation of the

diurnal expression of GSL pathway genes. Loss of function of *FRS7* and *FRS12* results in plants with increased expression of GLS biosynthetic genes. Interestingly, the observed increases in GSL biosynthetic gene expression occurred in a time of day-dependent manner, supporting the involvement of FRS7 and FRS12 in the diurnal regulation of the GSL pathway. Furthermore, we showed that combined, FRS7 and FRS12 are potentially able to physically bind to 20 promoters distributed among the indolic and aliphatic branches of the GSL pathway, at least in yeast. We further demonstrated that FRS7 and FRS12 physically interact with co-repressors of the JA signaling pathway such as JAZ4, JAZ6, JAZ8 and NINJA, suggesting the connections between these TFs and JA. NINJA bridges JAZ proteins to the general co-repressor TPL and thereby contributes to the repression of MYC TF activities (Pauwels *et al.*, 2010). Loss of function of *NINJA* results in a constitutive activation of the JA response in Arabidopsis (Acosta *et al.*, 2013). Considering that FRS7 and FRS12 preferentially act as transcriptional repressors, NINJA is likely recruited to build repressor complex(es). Furthermore, our study suggests multiple functions of NINJA in the regulation of defense-related genes; *i.e.* not only as part of the canonical JA signaling module, as previously established, but also as part of another complex with FRS7 and FRS12. This hypothesis was further enforced at the genetic level when the *frs7-1;frs12-1* allele was introgressed in the *ninja-1* allele. In the resulting triple *frs7;frs12;ninja* line, GSL biosynthetic genes were more strongly upregulated as compared to either of its parental lines, suggesting the additive deregulation of the GSL pathways, most presumably as a consequence of the deregulation of both the FRS7–FRS12–NINJA and MYC–JAZ–NINJA modules. Importantly, these expression phenotypes were also supported at the metabolite level. Finally, the fact that FRS7 and FRS12 can interact with JAZ proteins suggests the potential direct modulation of JA in the activities of these TFs. This hypothesis remains to be supported with further experimentation however. The FRS7 and FRS12 proteins belong to a family of transposase-derived TFs. The founding members of this family are the FAR-RED ELONGATED HYPOCOTYLS3 (FHY3) and its homolog FAR-RED IMPAIRED RESPONSE1 (FAR1), which both have crucial functions in plant growth and development, including light signaling and circadian clock entrainment. Recently, these TFs have been shown to modulate the growth–defense balance by recruiting JAZ and MYC2 proteins and thereby repressing defense-related genes in shade conditions (Liu *et*

al., 2019). These studies together with ours point out the function of FRS TFs as light-related modulators of the JA signaling pathway. Finally, we have previously shown that FRS7 and FRS12 particularly accumulate during short days to regulate clock outputs (Ritter *et al.*, 2017). It is therefore likely that the repression exerted by FRS7 and FRS12 on the GLS pathway is accentuated when the days are short, which could constitute a mechanism to prevent the overinduction of plant defense during short days. Further analysis will therefore be needed to establish how different photoperiods may affect the activity of NINJA-containing repressor complexes in the regulation of plant defense processes such as GSL biosynthesis.

Acknowledgments

We thank Annick Bleys for help in preparing the manuscript, Frederik Coppens and Veronique Storme for assistance in the statistical and bioinformatics analyses and Debora Gasperini for providing the *ninja-1, ProNINJA:GUSplus* and *ProNINJA:NINJA-CIT* lines. This study was funded by the Belgian Science Policy organization with postdoctoral fellowships to A.R and S.I., by the Research Foundation Flanders with postdoctoral fellowships to L.P. and P.F-C., with research project grants to A.G (G005212N, G005312N and G005915N) and L.P. (1507013N) and by the Marie Curie Actions program FP7-PEOPLE-2013-IEF for a postdoctoral fellowship to P.F-C.

Author contributions

P.F-C., S.I., S.M.B., L.P., A.G. and A.R. designed the research., P.F-C., S.I., G.G., M.T., B.L., A.N.D., R.V.B., R.D.C. and A.R. performed experiments. D.E. and A.R. performed bioinformatics analysis. P.F-C., S.I., K.G., G.D.J, G.G. D.E., L.P., A.G. and A.R. interpreted results. P.F-C., S.I., D.K., A.G. and A.R. wrote the article. . P.F-C. and SI contributed equally to this work.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1. Loss of *FRS7* and *FRS12* function results in GSL biosynthetic gene upregulation.

Fig. S2. GLS biosynthetic genes are redundantly regulated by FRS7 and FRS12 in a time of day-dependent manner.

Fig. S3. Negative controls of the BiFC analysis (Fig. 2c) of FRS7, FRS12 and NINJA in *N. benthamiana* leaves.

Fig. S4. FRS7 and FRS12 do not interact with ABI5-binding proteins.

Fig. S5. GUS histochemical analysis of the spatial expression of *FRS7*, *FRS12* and *NINJA* in 14-day-old seedlings grown in SD conditions.

Fig. S6. Diurnal expression of FRS7 and FRS12 proteins in seedlings grown in SD conditions.

Fig. S7. Hierarchical clustering analysis of DEGs (LFC>1) compared to wild type in *frs7-1;frs12-1*, *ninja-1* and *frs7-1;frs12-1;ninja-1* mutants grown in SD conditions and harvested at ZT8.

Table S1. Primers used in this study.

Table S2. Differentially expressed genes in the *frs7-1;frs12-1* line in SD conditions at ZT8.

Table S3. Differentially expressed genes in the *frs7-1;frs12* line in SD conditions at ZT16.

Table S4. Protein identification details of the NINJA (bait) interactors obtained by TAP and identified by MS with the LTQ Orbitrap.

Table S5. Clusters of DEGs (LFC>1) and GO enrichment analysis in the *frs7-1;frs12-1*, *ninja-1* and *frs7-1;frs12-;ninja-1* lines.

Table S6. GSL levels measured in 14-day-old seedlings (μg/g) of the indicated genotypes grown under SD and LD conditions and harvested at ZT8.

FIGURE LEGENDS

Fig. 1. FRS7 and FRS12 regulate glucosinolate metabolism genes in Arabidopsis. **(a)** Heat maps representing genes identified by RNA-Seq as differentially regulated (log fold change (LFC) ≥ 2) in the *frs7-1;frs12-1* line compared to the Col-0 wild type grown in SD conditions harvested at ZT8 and ZT16. Genes are grouped by biological function categories. **(b)** Gene expression analysis by RT-qPCR of GLS biosynthetic genes in Col-0 wild type (wt) compared to *frs7-1;frs12-1* seedlings growing in short-day (SD) conditions and harvested at ZT0, 8, 16 and 24. Values represent the average expression of three biological replicates ± SEM; *P<0.05, Student's *t*-test; "1" represents the highest level of expression for a particular gene.

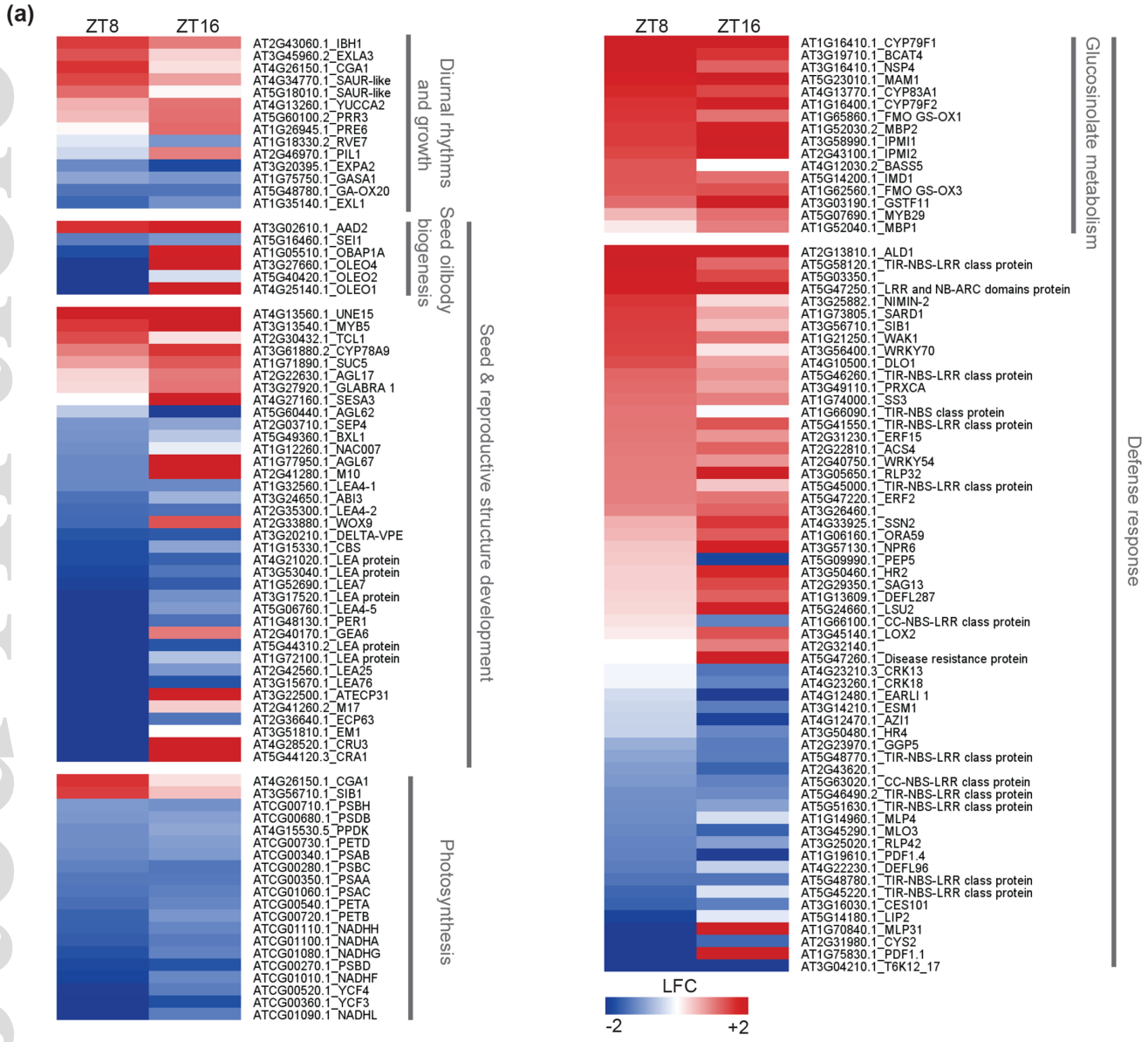
Fig. 2. FRS7 and FRS12 recruit the co-repressor NINJA. **(a)** Y2H assay using FRS7 and FRS12 as baits to test for protein interactions with characterized transcriptional regulators of the GSL pathway. AD, GAL4 activation domain; DBD, GAL4 DNA-binding domain. **(b)** Spoke model of the protein–protein interaction network identified with NINJA (pink node, bait) by TAP-MS in Arabidopsis cell cultures. The solid edges represent confirmed direct interactions and dashed edges unconfirmed interactions with prey proteins (purple nodes). **(c)** BiFC analysis in *Nicotiana benthamiana* leaves of nuclear interactions between NINJA and FRS7 or FRS12. Scale bars: 50 µm. **(d)** Y2H assay using NINJA as bait against other FRS family proteins as preys. **(e)** Mapping of the NINJA domain responsible for its interaction with FRS12. The numbers between brackets represent the amino acid lengths of the baits **(f)** Mapping of the FRS12 domain responsible for its interaction with NINJA. Transformed yeasts were spotted in tenfold dilutions on SD-LW control medium (-2) or SD-LWH selective medium (-3). Strains co-transformed with an empty vector were used as control for autoactivation.

Fig. 3. *NINJA* shows temporal co-expression with *FRS7* and *FRS12*. **(a)** Immunoblot analysis showing the diurnal contents of CIT-tagged NINJA protein in short-day (SD)-grown *ProNINJA:NINJA-CIT* Arabidopsis plants. MW, molecular weight. White and black regions indicate the light and dark periods, respectively. **(b)** Co-immunoprecipitation assay in plants expressing *NINJA-CIT* and *FRS7-HA* or *FRS12-HA* under regulation of

their respective endogenous promoters. Lines were grown in SD conditions and pre-incubated at ZT6 with cycloheximide for 1 h, followed by mock or 50-μM JA treatment for 15 or 30 min.

Fig. 4. FRS7, FRS12 and NINJA jointly regulate GSL biosynthetic genes in Arabidopsis. **(a)** Heat map representing cluster 5 of GSL biosynthetic genes identified as commonly upregulated in the *frs7-1;frs12-1, ninja-1* and *frs7-1;frs12-1;ninja-1* lines in short-day (SD) conditions at ZT8. Cluster 5 was identified by HCA of RNA-Seq transcriptomes of mutant lines and wild type. Rectangles highlight GSL biosynthetic genes. Values are represented as log fold-change (LFC) between mutant lines and wild type. **(b)** Diurnal oscillations of *CYP83A1* and *CYP79F1* steady-state transcript levels in wild type, *frs7-1;frs12-1, ninja-1* and *frs7-1;frs12-1;ninja-1* mutants grown in SD conditions. Gray rectangles represent the dark period. Values represent the average expression of three biological replicates ± SEM (*P<0.05, Student's *t*-test). **(c)** Interaction network of FRS7 and FRS12 TFs (purple nodes) and GSL-pathway promoters (blue nodes) identified by Y1H analysis. Gray edges represent bound promoters, and blue edges represent bound promoters and transcriptionally regulated genes.

Fig. 5. FRS7, FRS12 and NINJA regulate GLS biosynthesis. **(a)** Heatmap representation of GSL metabolites showing significant variations (P<0.05; 1-way ANOVA test) between wild-type and *ninja-1*, *frs7-1;frs12-1* and *frs7-1;frs12-1;ninja-1* 14-day-old Arabidopsis seedlings grown in short-day (SD) photoperiods and harvested at ZT8. Values are represented as log fold-change (LFC). **(b)** Accumulation of indolic GSL metabolites (glucobrassicin, 4-hydroxyglucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin) measured in wild-type, *ninja-1*, *frs7-1;frs12-1* and *frs7-1;frs12-1;ninja-1* 14-day-old Arabidopsis seedlings grown in SD photoperiods and harvested at ZT8. Values represent the average of five biological ± SEM. *P<0.05, **P<0.01, Student's *t*-test, comparison between *ninja-1*, *frs7-1;frs12-1* or *frs7-1;frs12-1;ninja-1* and wild type. #P<0.05, Student's *t*-test, comparison between *frs7-1;frs12-1* and *frs7-1;frs12-1;ninja-1*; °P<0.05, Student's *t*-test, comparison between *ninja-1* and *frs7-1;frs12-1;ninja-1*.



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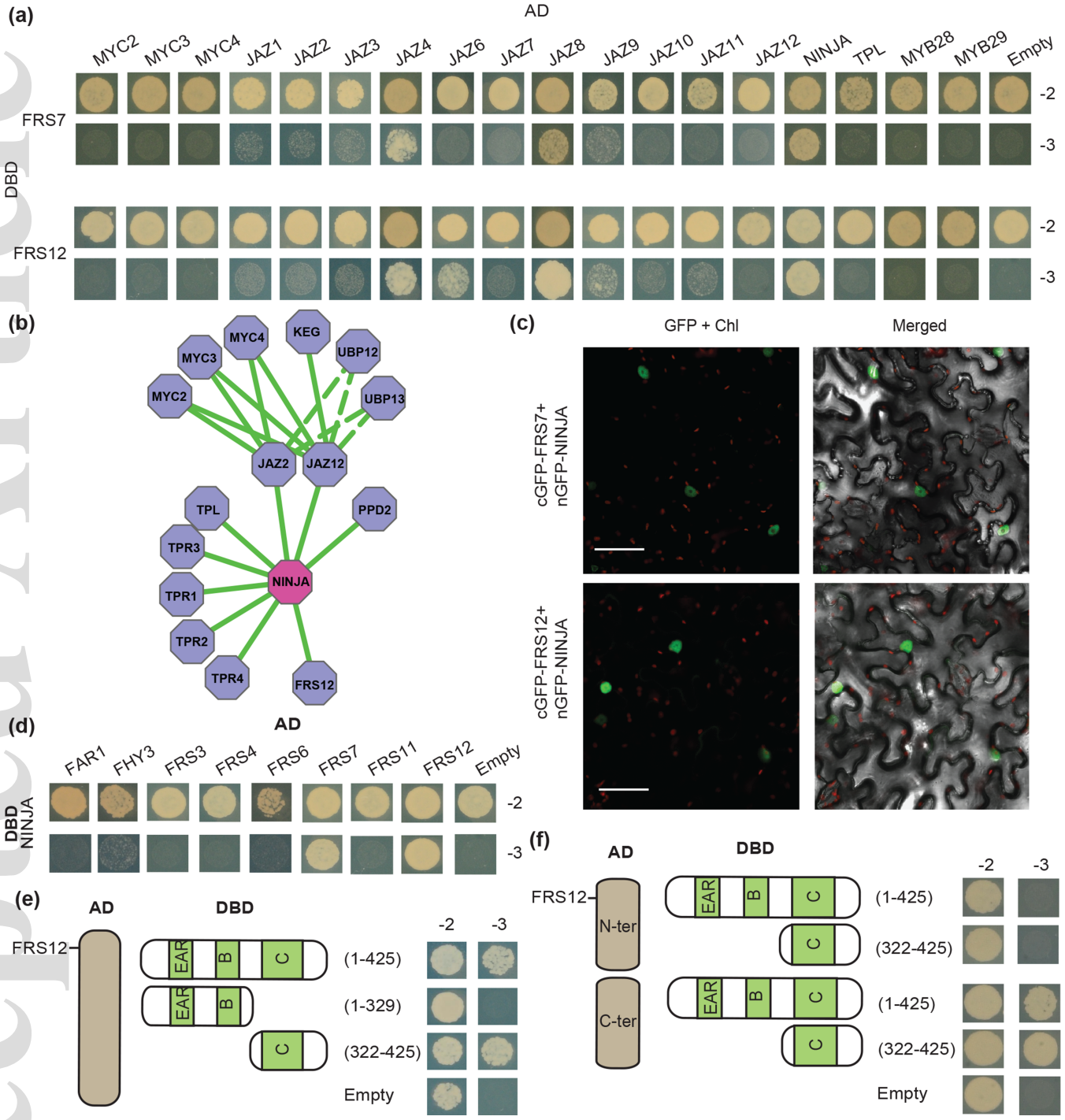

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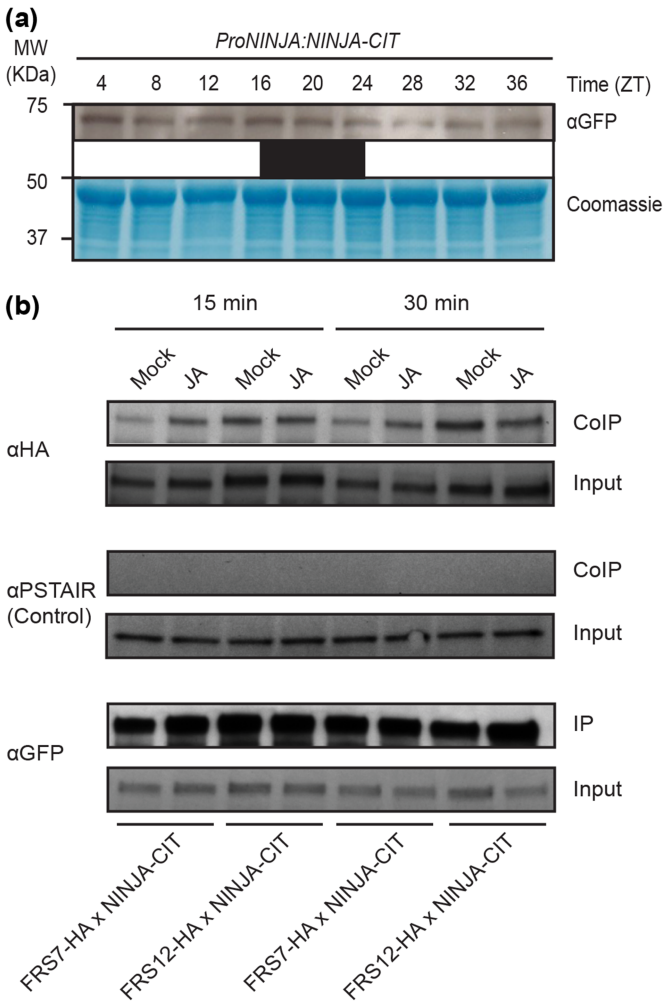

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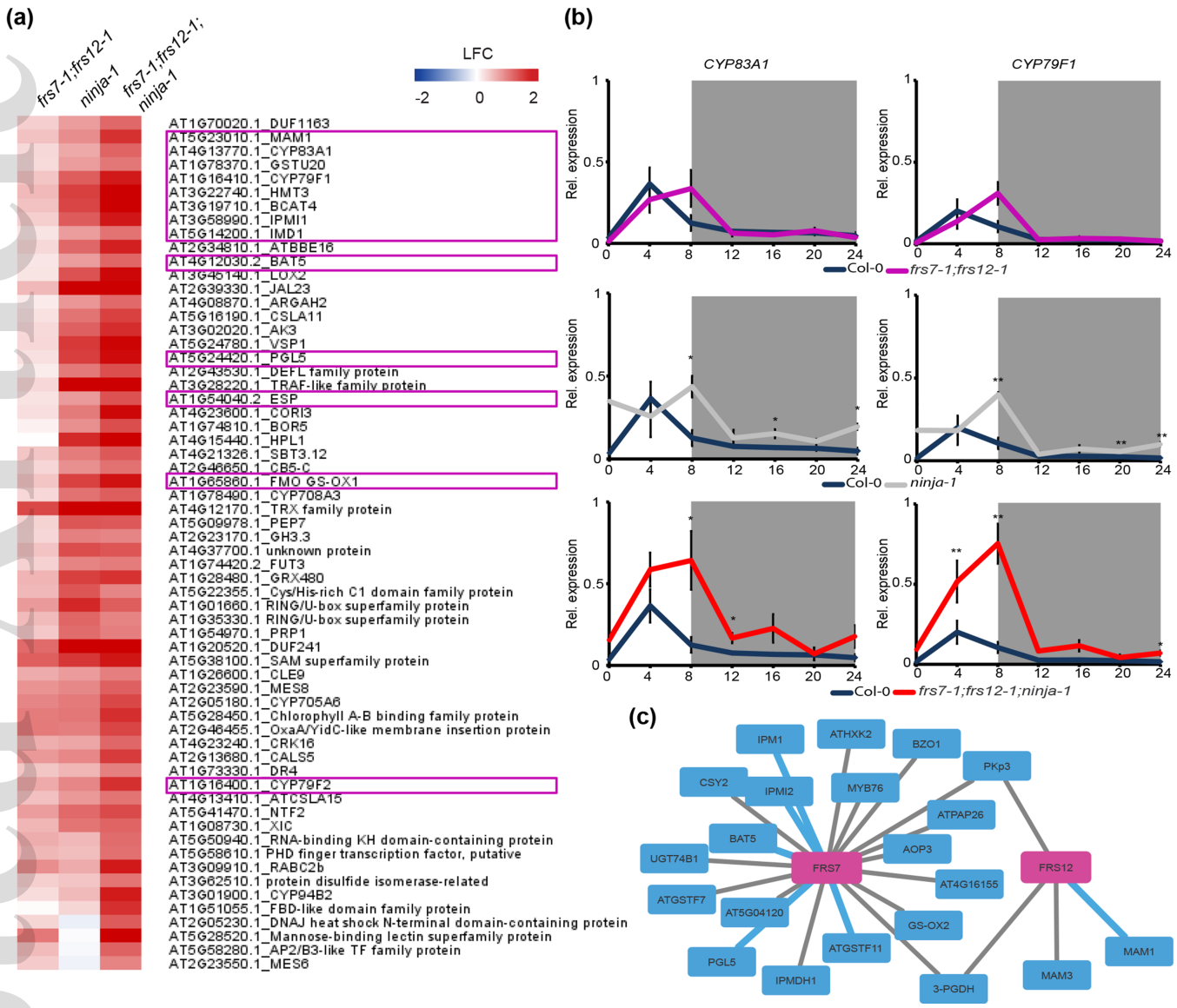

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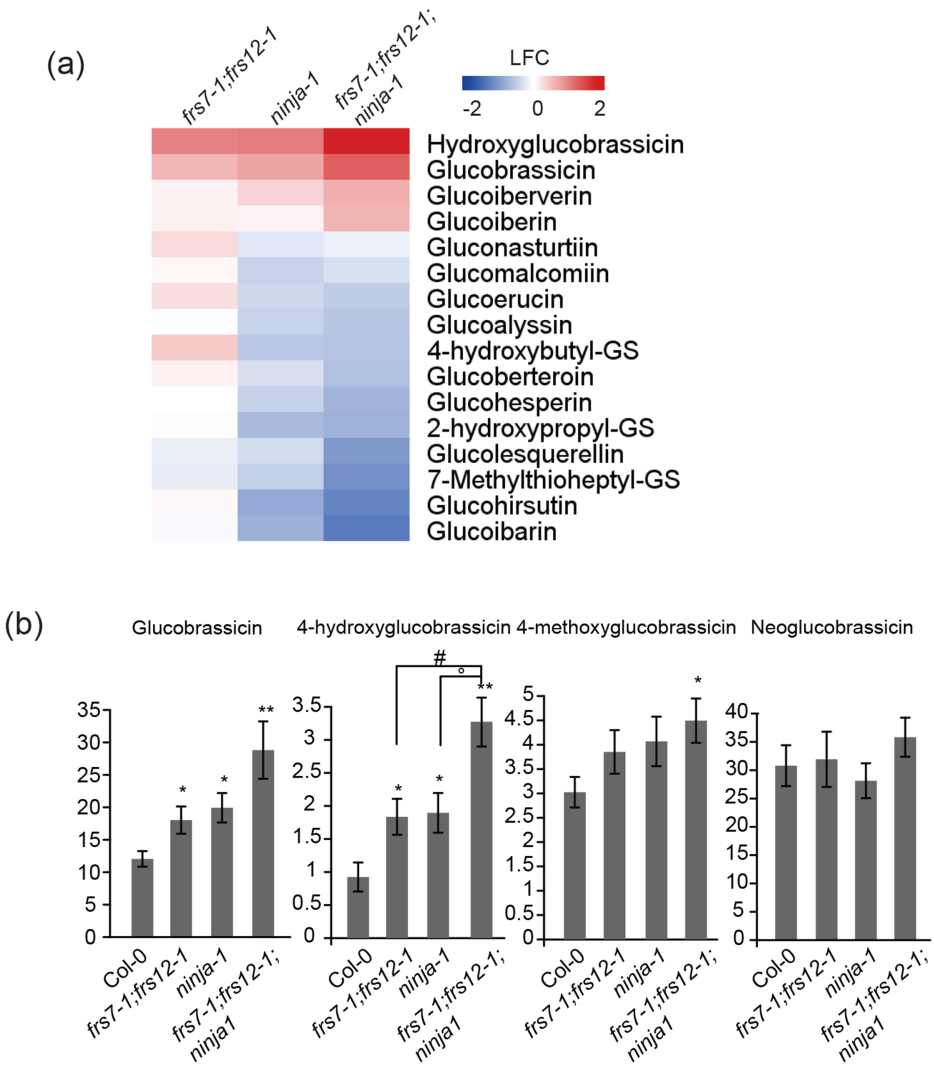

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